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Excision products of the T cell receptor gene support a progressive rearrangement model of the α/δ locus

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We have cloned extrachromosomal circular DNAs containing T cell receptor (TCR) δ gene segments in adult mouse thymocytes and splenocytes. We find that the frequency of circular DNA clones carrying germline δ sequences is lower than that of Jα probe-positive clones, possibly related to increasing 5′ distance from the most upstream Jα segment. This suggests that the TCR α/δ locus is successively rearranged from within and that the δ-containing excision products are progressively diluted out by the subsequent cell division which includes further α gene rearrangements. In addition, examination of δ gene excision products revealed newly identified Vδ subfamilies, the reciprocal joining of two Dδ elements, Jδ_2 usage in thymocytes and novel sequences homologous to the human δ-gene deleting elements.
Key words: circular DNA/D−D joining/δ-inactivating elements/Jδ_2 usage/new Vδ

Introduction

Immunocompetent T cells are generated in the thymus and eventually emigrate to the peripheral lymphoid tissues such as the spleen and lymph nodes (Weissman, 1967; Owen and Raff, 1970; Scollay, 1982). Development in the thymus is characterized by both rapid proliferation and cell death (Scollay *et al.*, 1984; Rothenberg and Lugo, 1985) which probably reflect the expression and selection of T cell receptors (TCRs) in the intrathymic microenvironment. There are two major types of TCR heterodimers:$\alpha\beta$ (Allison *et al.*, 1982; Haskins *et al.*, 1983; Meuer *et al.*, 1983) and $\gamma\delta$ (Brenner *et al.*, 1986; Lew *et al.*, 1986). In the adult mouse, TCR $\gamma\delta$ is expressed on a small fraction of thymocytes (Lew *et al.*, 1986), peripheral T cells (Cron *et al.*, 1988), dendritic epidermal cells (dECs) (Bonyhadi *et al.*, 1987; Koning *et al.*, 1987) and intestinal intraepithelial lymphocytes (Bonneville *et al.*, 1988). During thymic development, TCR $\gamma\delta$-bearing cells appear before those bearing $\alpha\beta$ (Bluestone *et al.*, 1987; Pardoll *et al.*, 1987). Like immunoglobulin (Ig) genes, TCR variable region genes are assembled from the separate germline variable (V), diversity (D) and joining (J) DNA segments during T cell differentiation. Rearrangement of TCR genes is mediated by a 'joining signal' adjacent to each germline V, D or J segment, consisting of a palindromic heptamer and an A + T rich nonamer signal sequence separated by a spacer of either 12 or 23 bp. These are located directly 3′ to each of the V gene segments, 5′ to the J gene segments and on either side of the D gene segments. Gene segments linked to joining signals with 12 bp spacers appear to recombine only with those linked to joining signals containing 23 bp spacers and vice versa (12/23 spacer rule; Early *et al.*, 1980; Sakano *et al.*, 1980). This DNA rearrangement generates two distinct classes of recombinant junctions. We refer to the junction between coding sequences as a coding joint (CJ) and the two joining signals fused head-to-head as the reciprocal joint (RJ) (Lewis *et al.*, 1984; 1985). The TCR α, β and γ genes are all unlinked, but the TCR δ gene is located within the TCR α locus, between Vα and Jα (Chien *et al.*, 1987a, b; Hata *et al.*, 1987; Loh *et al.*, 1987; Elliott *et al.*, 1988; Takihara *et al.*, 1988). Analysis of the direct rearrangements showed that two types of rearranged genes, TCR α and δ are differentially generated from this complex locus, suggesting that the recombination system at the $\alpha-\delta$ complex locus plays a key role in determining T cell lineages during thymic development.

Recently, we (Fujimoto and Yamagishi, 1987; Toda *et al.*, 1988) and others (Okazaki and Sakano, 1988; Okazaki *et al.*, 1987) have isolated the excision products of TCR α and δ, and TCR β gene rearrangements enriched in extrachromosomal circular DNA molecules from thymocytes. Previous electron microscopy of thymocyte circular DNA (Yamagishi *et al.*, 1982, 1983; Fujimoto and Yamagishi, 1987; Fujimoto *et al.*, 1985) showed the polydisperse distribution in size from 0.6 to >150 kb with a mean length of 16.7 kb, which is far less than the distance spanning Vα and Jα. These results raise the question of whether small circular DNAs are generated by successive recombination events preceding the productive α gene rearrangements or whether they are processed from initially excised large α circles. If a majority of circular DNAs came from the primary large α circles, representation of the δ locus in the circular DNA clones should be higher than the α locus. In successive recombination events, δ circles excised first may be diluted out during cell propagation with the occurring further α gene rearrangements. We found a low frequency of δ probe-positive circular DNA clones including a germline sequence compared with the α-positive clones. In order to show the excision product of successive gene rearrangements of TCR α/δ locus, we have characterized the circular DNAs in young adult thymocytes undergoing intrathymic selection compared with mature peripheral T cells in the spleen. Here, we confirm the distinct adult pattern of TCRδ endogenous gene rearrangements which show extensive diversity. Moreover, we show a murine δ gene-inactivating recombination system which may precede TCR α gene rearrangements and downregulate TCR δ chain formation in maturing thymus. Together, these results favor the concept of progressive gene rearrangements in the TCR α/δ locus.

Table I. Plaque hybridization of circular DNA clones

Mouse strain	Source	No. of clones screened	Probes						mtDNA clones (%)
			ΨJα−Jα$_1$	Dβ$_{1,2}$	Jγ$_1$	Dδ$_2$−Jδ$_1$	Jδ$_2$	δRec1	
C57BL/6	4WT	2.4 × 10^5	(697)	231	179	51	26	ND	18.5
	8WT	2.0 × 10^5	(1175)	1093	1183	162	158	ND	19.0
	4WS	1.5 × 10^5	(426)	72	171	25	41	ND	13.7
	8WS	1.0 × 10^5	(379)	282	280	ND	ND	ND	20.0
	8WS	7.7 × 10^5	ND	ND	ND	124	132	ND	ND
	8WS	1.6 × 10^5	156	828	713	36	20	9	ND
BALB/c -nu/nu	8WS	2.0 × 10^5	(61)	1	6	2	0	ND	24.5
	8WS	2.5 × 10^5	ND	ND	ND	2	3	ND	ND

Probe-positive clones were scored. Probe used: ΨJα−Jα$_1$, 8.0 kb *Eco*RI fragment of TA28.1 for the score in parenthesis and the 3.5 kb *Eco*RI−*Hin*dIII fragment (Figure 1); Dβ$_1$, 1.8 kb *Pst*I fragment (HG77); Dβ$_2$, 2.4 kb *Hin*dIII−*Eco*RI fragment (HG78) (Kronenberg *et al.*, 1985); Jγ$_1$, 2.6 kb *Eco*RI−*Hin*dIII fragment (pTG1) subcloned from 15 kb *Eco*RI fragment (pBCγ711) (Hayday *et al.*, 1985); Dδ$_2$−Jδ$_1$, 2.3 kb *Sac*I fragment (pCDS26); Jδ$_2$, 543 bp fragment (pCDS85) prepared from pCDS15 by deleting the 5′ region; δRec1, 1.15 kb *Kpn*I−*Pst*I fragment with driver DNAs (Figure 1). Percentage of mitochondrial DNA clones was measured with the probes of 11 kb and 5.2 kb *Bam*HI fragments of rat mitochondrial DNA cloned in pBR322 (Kobayashi and Koike, 1979). 4WT, 4 week old mouse thymocyte; 8WT, 8 week old mouse thymocytes; 4WS, 4 week old mouse splenocytes; 8WS, 8 week old mouse splenocytes. ND, not determined.

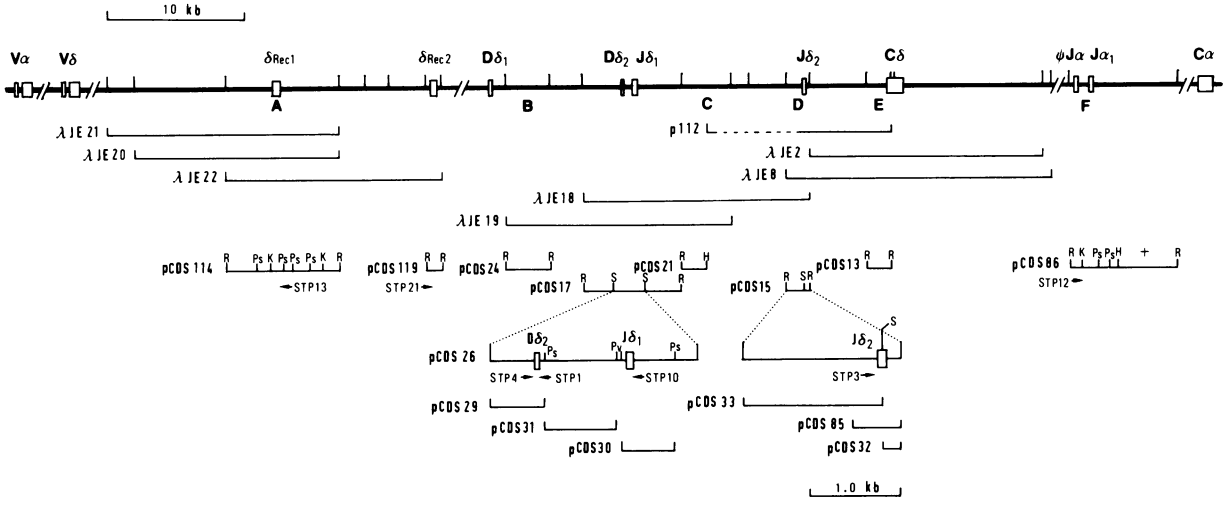

Fig. 1. Genomic organization of the TCR α/δ locus and the location of germline clones used. Vertical bar on the top line represents *Eco*RI site. Abbreviations: R, *Eco*RI; K, *Kpn*I; H, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*II; S, *Sac*I; +, unassigned restriction sites. STP1, 3, 4, 10, 12, 13 and 21 show the orientation of sequencing primer. Primers used for sequencing the germline segments: AAAGAGCAAAGTCATTGCC (STP12), AGCAGCTGTGGGTTTATGC (STP13) and GAATATGGATCAGTCAATT (STP21).

Results

Circular DNA clones containing TCR gene loci

In order to examine the excision products of TCR gene rearrangements, we isolated circular DNAs from murine (C57BL/6) thymocytes and splenocytes and cloned them into the *Eco*RI site of λgt11 phage vector. Typically, we obtained 0.4−3 × 10^6 recombinant phages/μg of vector DNA as described in Materials and methods.

The phage library of circular DNA was screened with TCR α (ΨJα−Jα$_1$), β (Dβ$_{1,2}$), γ (Jγ$_1$) and δ (Dδ$_2$−Jδ$_1$ and Jδ$_2$) gene probes (Table I). Since germline *Eco*RI fragments of ΨJα−Jα$_1$ (8.0 kb), Dβ$_1$ (9.0 kb), Jγ$_1$ (15.0 kb) and Dδ$_2$−Jδ$_1$ (7.4 kb) are excluded from this library due to the upperlimit of packaging capacity (7 kb), most of these probe-positive clones may be rearranged. The frequency of every TCR probe-positive clone was similar in both thymocytes and splenocytes. The low frequency of TCR probe-positive clones from splenocytes of athymic nude mice serves as a relevant control emphasizing that the excision products are thymus-dependent TCR gene rearrangements from cells which survived as euthymic mouse splenocytes.

Rearrangement status of TCR δ locus in αβ-bearing T cells

A germline sequence of the δ segment should only appear in the circular DNA library as a resulting recombination occurring at the flanking regions. We chose to analyze four packageable germline sequences; including a 3.2 kb *Eco*RI fragment between Dδ$_1$ and Dδ$_2$ (B), a 3.5 kb *Eco*RI fragment between Jδ$_1$ and Jδ$_2$ (C), a 1.7 kb *Eco*RI fragment of infrequently rearranged Jδ$_2$ (D), a 1.8 kb *Eco*RI fragment upstream of Cδ (E) and two oversized germline sequences of *Eco*RI fragment (A and F) (Figure 1). The frequency at which we found each segment in splenic circular DNAs decreased with the 5′ distance from the most upstream Jα segment, irrespective of the rearrangement status (Table II). We further analyzed the rearrangement status of a single 8.0 kb *Eco*RI fragment containing Jα$_1$ (Table III). Again, the number of DNA clones observed which originated upstream of Jα$_1$ was fewer than those derived from downstream of Jα$_1$. The low frequency of circular DNA clones containing segments distal to Cα cannot be explained by a single excisional event involving the α circles or by second-

ary rearrangement of an excised circular DNA. Instead, these data favor the occurrence of successive gene rearrangements in which the preceding δ gene excision products have been diluted out during subsequent cell division that included additional α gene rearrangements.

Table II. Appearance of a germline segment (B to E) in circular DNA clones

Probes	Driver DNA	Size of germline *Eco*RI-fragment (kb)	Location	No. of clones
δRec1	+	8.3	A	9
pCDS24	−	3.2	B	9
pCDS21	+	3.5	C	18
$Jδ_2$	−	1.7	D	21
pCDS13	+	1.8	E	28
$ΨJα-Jα_1$	−	8.0	F	69

8 weeks old mouse splenocyte circular DNA library (2.0×10^5 clones) was screened by plaque hybridizations. Probes used: $ΨJα-Jα_1$ (3.5 kb *Eco*RI−*Hin*dIII fragment), $Jδ_2$ and δRec1 are described in Table I; pCDS24 (3.2 kb *Eco*RI fragment), pCDS21 (1.7 kb *Eco*RI−*Hin*dIII fragment) and pCDS13 (1.8 kb *Eco*RI fragment) are shown in Figure 1. Location of each fragment is shown by a letter A to F in Figure 1.

Table III. Analysis of circular DNA clones positive to a $ΨJα-Jα_1$ segment (F)

Probes			No. of positive clones	Predicted recombinant structure
ΨJα	$Jα_1$	3′−$Jα_1$		
+	−	−	27	RJ (V, δRec, D)−ΨJα
−	+	−	11	RJ V−$Jα_1$ + CJ (V, δRec, D)−ΨJα
+	+	+	23	RJ V−Jαx
−	+	+	1	RJ V−Jαx + CJ V−$Jα_1$
−	−	+	41	RJ V−Jαx + CJ V−Jαx

103 positive clones from 8 week old mouse splenocyte circular DNA library were further analyzed by plaque hybridizations. Probes were prepared from pCDS86 (Figure 1): ΨJα, 1.0 kb *Eco*RI−*Kpn*I fragment containing ΨJα; $Jα_1$, 1.5 kb *Kpn*I−*Pst*I fragment containing $Jα_1$; 3′ $Jα_1$, 0.8 kb *Pst*I fragment downstream of $Jα_1$. Distance between ΨJα and $Jα_1$ is shortened by a strain-dependent deletion compared with the other strain (Std:ddY). RJ, reciprocal joint. CJ, coding joint. Jαx, uncharacterized Jαs 3′ to $Jα_1$.

Identification of TCR δ gene rearrangements in circular DNA clones

In order to analyze TCR δ gene rearrangement in more detail, we used a circular DNA clone, p112 isolated from 4 week old mouse (Std:ddY) thymocytes (Toda *et al.*, 1988). This clone contains a 7.3 kb germline sequence of a $Jδ_2-Cδ$ intron with a strain-dependent deletion of 6.4 kb and represents the starting point of the chromosomal walk to isolate four genomic DNA clones of *Eco*RI partial digests extending further 5′ (Figure 1). To characterize the DNA rearrangements of TCR δ probe-positive clones, we prepared five subclones: 5′ $Jδ_2$ (pCDS33) and 3′ $Jδ_2$ (pCDS32) for $Jδ_2$ probe-positive clones; 5′ $Dδ_2$ (pCDS29), $Dδ_2-Jδ_1$ (pCDS31) and 3′ $Jδ_1$ (pCDS30) for $Dδ_2-Jδ_1$ probe-positive clones. Of $Jδ_2$ probe-positive clones, we chose 12 each from 4 week and 8 week old adult thymocytes, and 24 clones from 8 week old adult splenocytes. In addition, 12 $Dδ_2-Jδ_1$ probe-positive clones of 8 week old adult splenocytes were selected and all these clones were analyzed by Southern hybridization with appropriate probes as shown in Table IV.

Three of 12 $Jδ_2$ probe-positive clones from 4 week old thymocytes and one of 12 clones from 8 week old thymocytes did not correspond in size to the *Eco*RI probe of germline $Jδ_2$ (pCDS15, 1.7 kb). Southern hybridizations with the $Jδ_2$ subclones derived from either the 5′ or 3′ region of germline $Jδ_2$ sequence predicted that at least two clones were rearranged. None of 24 $Jδ_2$ probe-positive clones from splenocytes of 8 week old mice were rearranged. Even more strikingly every $Dδ_2-Jδ_1$ probe-positive clone examined showed a smaller molecular size than the *Eco*RI fragment of the germline $Dδ_2-Jδ_1$ fragment (pCDS17, 7.4 kb), suggesting that these clones corresponded to rearranged structures. To evaluate these clones further, nucleotide sequencing was performed using synthetic specific

Table IV. Southern hybridization analysis of circular DNA clones

Clones	Source	Size (kb)	Probes					Predicted recombinant structure	Sequencing primer used
			pCDS33	pCDS32	pCDS29	pCDS31	pCDS30		
pCDS109	4WT	1.5	+	+	ND	ND	ND	deletion outside J_2	STP3, M4, RV
110	4WT	5.4	+	−	ND	ND	ND	RJ (VD)−J_2	STP3, M4, RV
111	4WT	1.4	+	+	ND	ND	ND	deletion outside J_2	STP3, M4, RV
112	8WT	3.3	−	+	ND	ND	ND	CJ (VD)−J_2	M4, RV
pCDS73	8WS	7.0	ND	ND	+	−	−	RJ V(δRec)−D_2 or D_1-D_2	STP4
74	8WS	7.0	ND	ND	+	−	+	CJ D_2-J_1	STP4, STP10
75	8WS	5.8	ND	ND	−	−	+	CJ (VD_1)D_2-J_1	STP10
76	8WS	3.0	ND	ND	+	−	−	RJ V−D_2 or D_1-D_2	STP4
77	8WS	3.0	ND	ND	+	−	−	RJ V−D_2 or D_1-D_2	STP4
78	8WS	3.0	ND	ND	+	−	−	RJ V−D_2 or D_1-D_2	STP4
79	8WS	3.0	ND	ND	+	−	−	RJ V−D_2 or D_1-D_2	STP4
80	8WS	6.0	ND	ND	−	+	+	CJ (V)D_1-D_2 or RJ D_2-J_2(ΨJα)	STP1
81	8WS	5.0	ND	ND	−	−	+	CJ (VD_1)D_2-J_1	STP10
82	8WS	3.0	ND	ND	+	−	−	RJ V−D_2 or D_1-D_2	STP4
83	8WS	5.8	ND	ND	−	−	+	CJ (VD_1)D_2-J_1	STP10
84	8WS	4.8	ND	ND	−	−	+	CJ V(δRec)D_2-J_1	STP10

TCR δ gene-positive phage clones were analyzed by Southern hybridizations with five different DNA probes and sequenced by using the universal M13 primer (M4, RV) or appropriate specific primers (Figure 1): CACGTGATACAAAGCCCAGGG (STP4, upstream of $Dδ_2$), GTTTACCTTCCATGGTGGC (STP1, downstream of $Dδ_2$), TTGGTTCCACAGTCACTTG (STP10, downstream of $Jδ_1$) and ACACAGATGTGAAACCCAG (STP3, upstream of $Jδ_2$). 4WT, 8WT and 8WS are as described in Table I. RJ and CJ are described in Table III. ND, not determined.

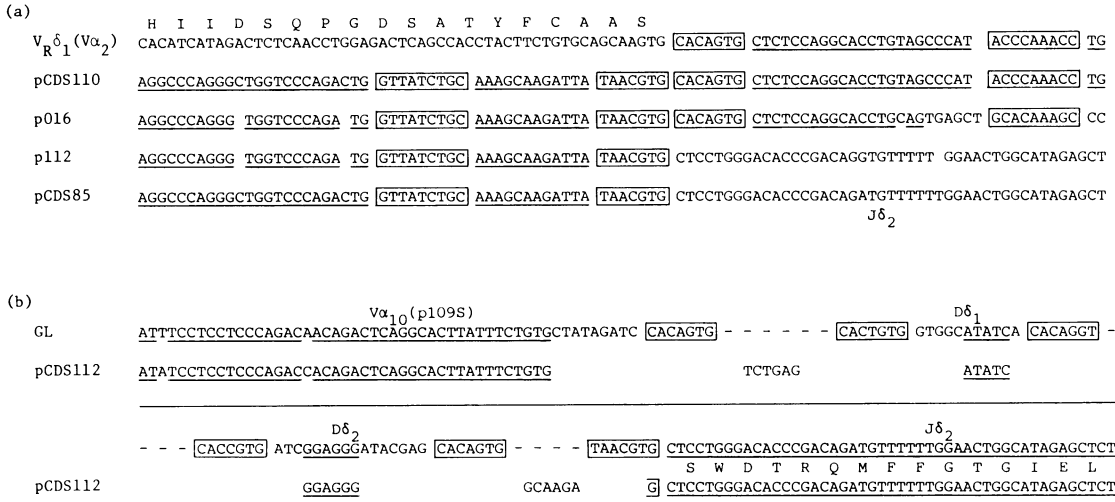

Fig. 2. Nucleotide sequences of circular DNA clones pCDS110 (**a**) and pCDS112 (**b**) from adult thymocytes. Signal sequences are boxed. Homologous sequences compared are underlined. Sequences compared: $V_R\delta_1$ (Okazaki and Sakano, 1988); $J\delta_2$, $D\delta_1$ and $D\delta_2$ (Chien *et al.*, 1987b); p016, p112 and p109S (Toda *et al.*, 1988); pCDS85 (Figure 1); GL, germline sequence. Difference of $J\delta_2$ coding sequence between p112 (Std:ddY) and pCDS85 (C57BL/6) may be due to the strain-dependent polymorphism.

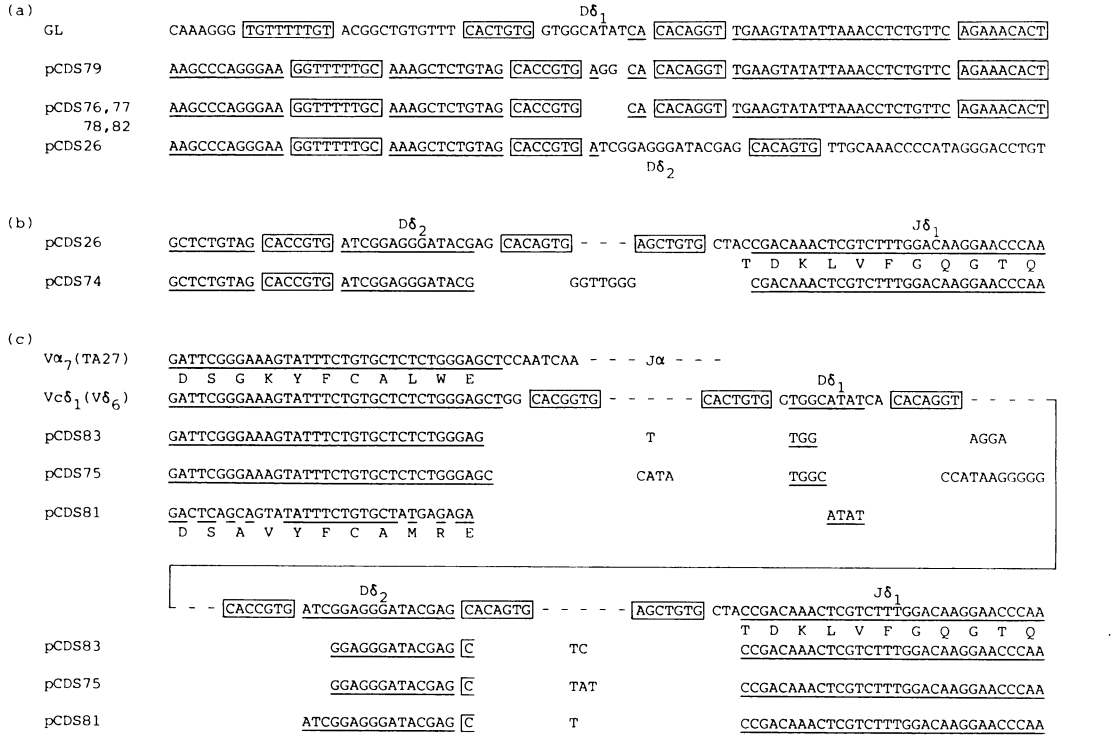

Fig. 3. Nucleotide sequences of circular DNA clones from adult splenocytes. (**a**) D−D RJ (pCDS79, 76, 77,78 and 82). (**b**) D−J CJ (pCDS74). (**c**) V−D−D−J CJ (pCDS83, 75, 81). Sequences compared: GL, germline sequence; $J\delta_1$ (Chien *et al.*, 1987b); pCDS26 (Figure 1); $V\alpha_7$ (Arden *et al.*, 1985); $Vc\delta_1$ (Okazaki and Sakano, 1988). Symbol indications are as described in Figure 2.

oligonucleotides as extension primers as shown in Table IV and Figure 1.

$J\delta_2$ gene rearrangements in adult thymocytes

We have sequenced four circular DNA clones pCDS109, 110, 111 and 112 which are $J\delta_2$ probe-positive (Figure 2). Clone pCDS110 contained a reciprocal V−$J\delta_2$ joint fused precisely. The RJ of p016 isolated previously from murine (Std:ddY) thymocytes (Toda *et al.*, 1988) is also very similar to the present pCDS110 RJ. The downstream region of the RJ was identical to the 3′ region of $V_R\delta_1$, a member of the $V\alpha_2$ family (Arden *et al.*, 1985), which had been used in the precise RJ of V−$J\delta_1$ joining (Okazaki and Sakano, 1988). The upstream region of the RJ was identical to the 5′ signal sequence of $J\delta_2$.

Clone pCDS112 contained a recombinant coding structure of V−$D\delta_1$−$D\delta_2$−$J\delta_2$ joining. The V region used was very similar to V_{109S}, a member of $V\alpha_{10}$, fused with $J\alpha_1$ (Fujimoto and Yamagishi, 1987; Toda *et al.*, 1988). Recently, a $V\alpha_{10}$ homolog has been shown to be utilized as $VDDJ_1$ in adult thymocytes (Takagaki *et al.*, 1989a) and intestinal intraepithelial lymphocytes (Takagaki *et al.*, 1989b). At the junctions, there were random nucleotide (N) sequence insertions including a single G derived from the

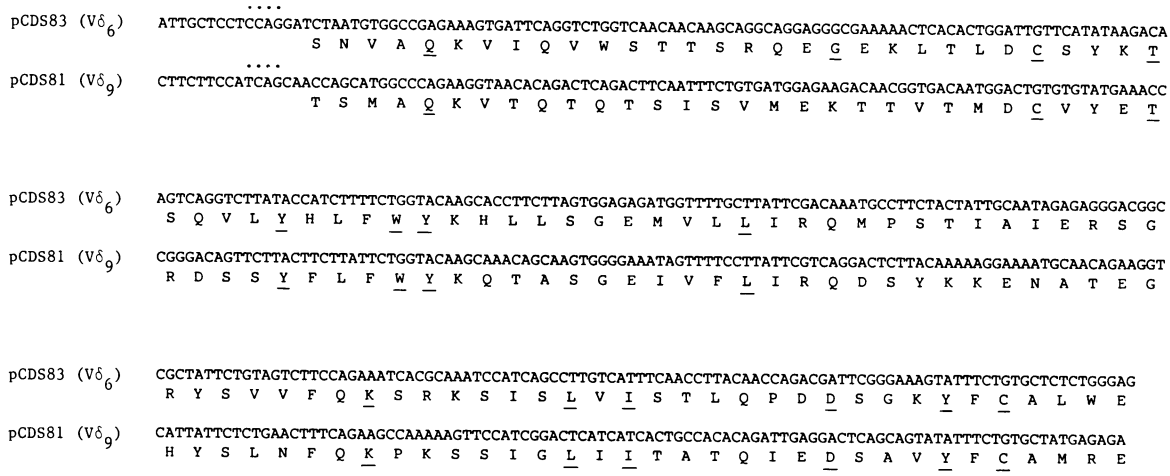

Fig. 4. Comparison of Vδ gene sequences rearranged in the circular DNA clones, pCDS83 ($V\delta_6$) and pCDS81 ($V\delta_9$). The sequences of pCDS83 and pCDS75 were identical. Amino acid residues conserved in Vδ genes are underlined. Splicing acceptor signals are overlined with dots.

5′ signal heptamer of $J\delta_2$, resulting in coding joint sequences that are out-of-frame. The simultaneous use of $D\delta_1$ and $D\delta_2$ elements and insertion of random nucleotides are a distinct feature of V gene assembly in adult thymocytes (Elliott *et al.*, 1988). The sequence analysis of pCDS109 and 111 revealed a germline configuration of $J\delta_2$, suggesting deletional recombination occurred outside of $J\delta_2$ in these clones.

Excision products of TCR $D\delta_1-D\delta_2$ joining in circular DNA clones from splenic T cells

Splenic T cells may contain the excision products of TCR δ gene rearrangements which preceded functional α gene assembly in thymocytes (Table I). Five of 12 $D\delta_2-J\delta_1$ probe-positive DNA clones from splenocytes contained the reciprocal joint of $D\delta_1-D\delta_2$ joining (Figure 3a). Usually, it was difficult to show the D−D joining by analyzing the rearranged sequence due to the deletion of D sequence at the junctions. Four clones, pCDS76, 77, 78 and 82 contained $D\delta_1-D\delta_2$ RJs with two residual germline-encoded bases between the fused heptamers. This probably results from imprecise cleavage by a putative recombinase, and not from random nucleotide insertions which may result from the activity of terminal deoxynucleotidyl transferase. Clone pCDS79 showed two random nucleotides which were inserted between the fused heptamer, as well as three residual germline encoded bases.

TCR (VD) $D\delta_2-J\delta_1$ CJs in circular DNA clones from splenic T cells

In splenic T cells, clones containing TCR δ gene CJs may have been formed by excision at the time of $V\alpha-J\alpha$ joining. Southern analyses predicted that five clones contained the $D\delta_2-J\delta_1$ CJ; a simple $D\delta_2-J\delta_1$ joint for pCDS74 (Figure 3b) and the further upstream rearrangements for pCDS75, 81, 83 and 84. Clone pCDS74 presents the first functional $D\delta_2-J\delta_1$ joint, competent for further site specific recombination. None of the $D-J\delta_1$ joinings so far identified has retained a conserved heptamer sequence at their 5′ end (Elliott *et al.*, 1988). Three clones, pCDS83, 75 and 81 showed the recombinant structure of $V-D\delta_1-D\delta_2-J\delta_1$ joining (Figure 3c). The V segment used for pCDS83 and 75 was identical to $Vc\delta_1$ (Okazaki and Sakano, 1988), which is similar to $V\delta_6$ (Elliott *et al.*, 1988), a member of $V\alpha_7$ (Arden *et al.*, 1985). Recently, $V\delta_6$ has been shown to be utilized by T cell hybridomas responsive to mycobacterial antigens (O'Brien *et al.*, 1989). The V segment used by pCDS81 encodes several amino acids which are conserved among Vδ genes, indicating that this clone encodes a newly identified Vδ gene segment (Figure 4). These CJ sequences are non-functional since pCDS83 and 81 are out-of-frame and pCDS75 has the termination codon at the $V-D\delta_1$ junction. One to 11 bases of N sequence (including a single C derived from the 3′ signal heptamer of $D\delta_2$ coding sequence) were inserted at every joint except $V-D\delta_1$ and $D\delta_1-D\delta_2$ joints of pCDS81. Another clone, pCDS84 showed the non-V segment fused with the $D\delta_2-J\delta_1$ coding sequence (Figure 5a). At the junctions, there were N sequence insertions including a single G derived from the 5′ signal heptamer flanking the $D\delta_2$ coding sequence.

Murine homologs of human δRec and ΨJα

We cloned and sequenced the germline component (pCDS119) homologous to the non-V sequence of pCDS84 (Figures 1 and 5a). It contained a sequence homologous (64% homology on the 5′ side of signal heptamer) to the human δ-deleting element, termed δRec (de Villartay *et al.*, 1987; 1988) followed by a 23 bp spacer signal sequence (Figure 6).

Based on Southern hybridization analyses (Table IV), the recombinant structure of pCDS73 is the reciprocal joint of $D\delta_2$ rearranged with an upstream segment. Sequence analysis revealed the precise head-to-head ligation of two signal heptamers (Figure 5b). The upstream region of the fused heptamers derives from the 5′ flanking region of $D\delta_2$. The downstream region of the fused heptamers was highly homologous to the 3′ flanking sequence of human δRec. We cloned the germline 8.3 kb *Eco*RI fragment (pCDS114) homologous to the downstream region of the fused heptamers (Figure 1). It contained a sequence 80% homologous to human δRec (Figure 6). Clone pCDS73 represents the excision product of $D\delta_2$ rearranged to the human δRec-equivalent segment located upstream from $D\delta_2$. Two sequences homologous to human δRec were identified: clones pCDS114 (termed δRec1) and pCDS119 (termed

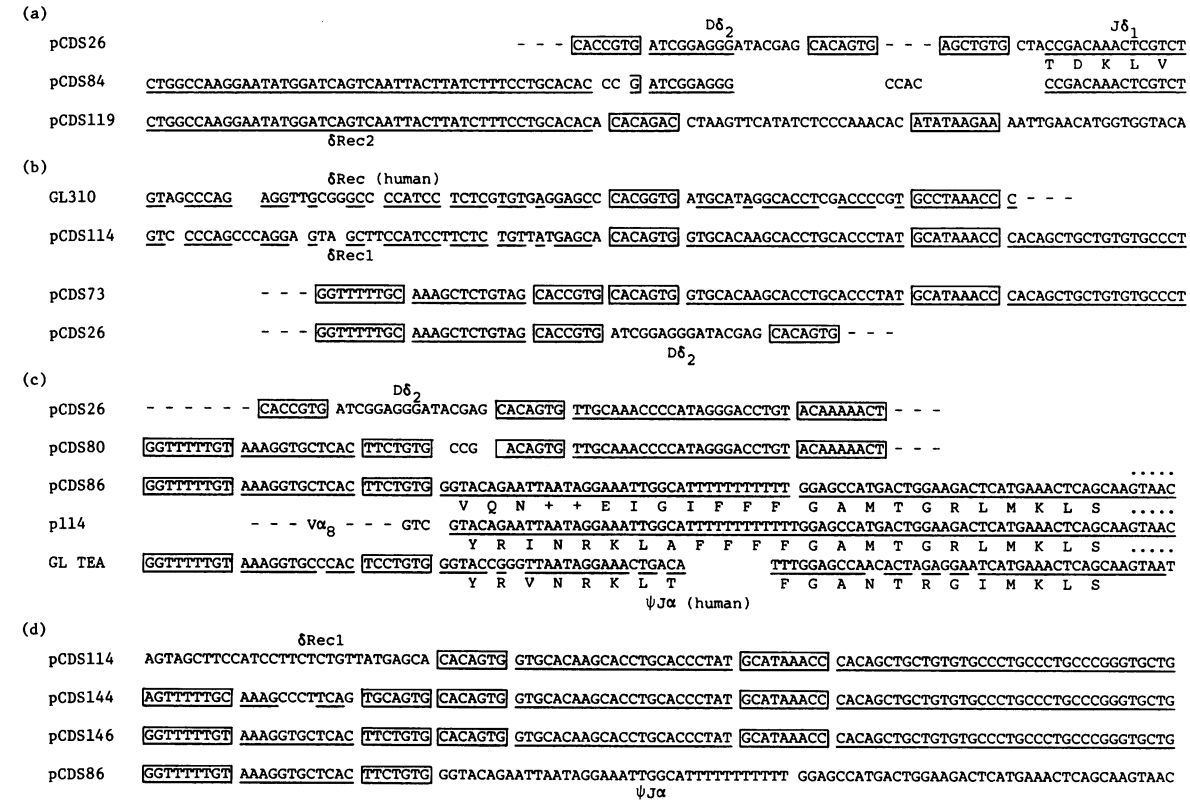

Fig. 5. Nucleotide sequences of circular DNA clones containing the deletional rearrangements of TCRδ. (**a**) δRec2−D_2−J_1 CJ (PCDS84). (**b**) δRec1−D_2 RJ (pCDS73). (**c**) D_2−ΨJα RJ (pCDS80). (**d**) δRec1−ΨJα (pCDS146) and δRec1−Jαx (pCDS144) RJs. Sequences compared: GL310 and GL TEA (de Villartay *et al.*, 1988); p114 (Toda *et al.*, 1988); pCDS26, pCDS86, pCDS114 and pCDS119 (Figure 1). Splicing donor signals are overlined with dots. Other indications are as described in Figure 2.

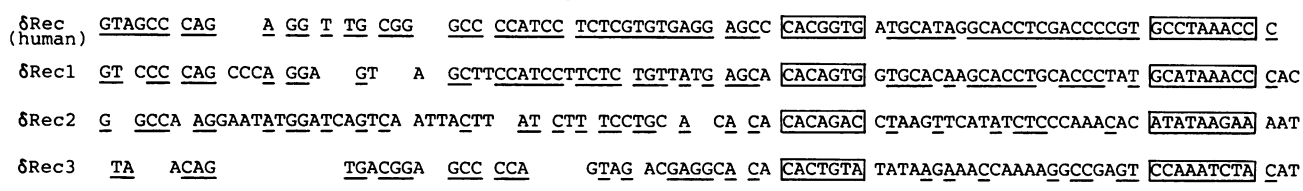

Fig. 6. Comparison of δRec sequences. δRec3 is from germline 3′ D_1 (Elliott *et al.*, 1988). Other indications are as described in Figure 2.

δRec2) which are 78% homologous on the 5′ side of their signal heptamers but which differ on their 3′ side (Figure 6). Moreover, these two δRec genes are linked on a germline fragment (Figure 1).

Southern hybridization analysis predicted that the recombinant structure of pCDS80 is either the (V)Dδ_1−Dδ_2 CJ or the RJ of Dδ_2 fused with the segment located downstream of Jδ_2 (Table IV). Sequence analysis revealed the truncated signal heptamers of the 3′ flanking region of Dδ_2 which was fused with the uncharacterized signal heptamer in a head-to-head fashion with a N sequence insertion (Figure 5c). In order to characterize the signal sequence upstream from the reciprocal joint, we have screened for the homologous germline component in the three overlapping cosmid DNA clones, TA28.1, TA8.1 and TA4.1, encompassing the 100 kb of DNA containing most of the Jα gene segments. We cloned and sequenced the homologous 8.0 kb *Eco*RI fragment (pCDS86) from TA28.1 (Figure 1). As shown in Figure 5c, the ΨJα sequence of the thymus circular DNA clone p114 (Toda *et al.*, 1988) was identified in the germline clone pCDS86. Only one nucleotide deletional change between two DNA clones (BALB/c and Std:ddY) may be attributable to strain-dependent polymorphism. The germline ΨJα sequence showed striking similarity (80% homology) to the human ΨJα of GL TEA, used as the receptor recombining with δRec. Moreover, both ΨJαs are mapped in a similar location between Cδ and the most upstream Jα segment (de Villartay *et al.*, 1988; Toda *et al.*, 1988). Thus, clone pCDS80 corresponds to the excision product of Dδ_2−ΨJα joining.

In order to assess whether δRec−ΨJα joining occurs in mice, as shown for human δ-chain deletional gene rearrangements (de Villartay *et al.*, 1987; 1988), we identified δRec1 and ΨJα-Jα_1 double positive clones in an amplified circular DNA library derived from 8 week old mouse splenocytes. Two clones, pCDS146 and pCDS144 showed the reciprocal joint of δRec1 recombined with ΨJα or uncharacterised Jα (possibly Jα_1) respectively (Figure 5d).

Discussion

New Vδ sequences

Circular DNA analysis of adult thymocytes by Okazaki and Sakano (1988) have shown that Vδ$_6$ (homologous to Vα$_7$) and a new Vδ (virtually identical to Vα$_2$ TA19) are used

both in α and in δ gene rearrangements. We also confirmed the usage of $V\alpha_7$ in the $V-D\delta_2$ coding joint in clone pCDS67 from fetal thymocytes (data not shown) and in the $V-D-D-J\delta_1$ coding joints in clones pCDS83 and 75 (Figure 3c), and the $V\alpha_2$ usage in the reciprocal joint of $V-J\delta_1$ joining in the clone pCDS67 (data not shown) and in the $V-J\delta_2$ joining characterized in clone pCDS110 (Figure 2a). The Vδ sequence used for the $V-D-D-J\delta_2$ coding joint of pCDS112 (Figure 2b) is virtually identical to the previously published $V\alpha_{10}$ sequence (Toda *et al.*, 1988). The Vδ sequence used for the $V-D-D-J\delta_1$ coding joint of pCDS81 (Figure 4) contains amino acids conserved in other Vδ gene families and has no precedent in association with Jα. Here, using the circular DNA isolation technique we identified three new Vδ gene sequences: a $V\alpha_{10}$-homolog termed $V\delta_7$ (pCDS112); a $V\alpha_2$-homolog termed $V\delta_8$ (pCDS67 and 110); and entirely new Vδ sequence termed $V\delta_9$ (pCDS81) (Figure 4). The $V\alpha_4$-homolog joined with $D\delta_2$ (Korman *et al.*, 1988) may be termed $V\delta_{10}$.

Variable V(D)J gene assembly

For TCR β and δ gene segments, both D−D joinings and direct V−J joinings skipping the D segments may be possible, in accordance with the 12/23 bp spacer rule (Early *et al.*, 1980; Sakano *et al.*, 1980). The RJs of circular DNA clones, pCDS76, 77, 78, 79 and 82 consisted of the 5′-$D\delta_2$ and 3′-$D\delta_1$ recombination signals, thus providing direct evidence for D−D joining. A circular DNA clone, pCDS110, contained a $V-J\delta_2$ RJ skipping the Dδ segment. Okazaki and Sakano (1988) have also shown the presence of a functional V−D−D coding joint and a $V-J\delta_1$ RJ on thymocyte circular DNA excised from a TCR δ gene. Analysis of DNA clones complementary to RNA from adult double-negative thymocytes has shown functional $V-D-D-J\delta_1$ rearrangements and aberrant $D\delta_1-J\delta_1$ rearrangements at the δ chain locus (Elliott *et al.*, 1988). In most cases, exonucleolytic degradation extends to the 5′ signal sequence of D segment joined to $J\delta_1$. A circular DNA clone, pCDS74 showed the first evidence of a $D\delta_2-J\delta_1$ joint which should be capable of serving as a substrate for further site-specific recombination. Analysis of rearranged genomic DNA clones isolated from fetal thymocytes showed the presence of functional joints of $V-D\delta_2-J\delta_1$, $V-D\delta_2$ and $D-D-J\delta_1$ at the δ chain locus (Chien *et al.*, 1987b). We also detected a $V\alpha_7-D\delta_2$ CJ and a $V\alpha_2-J\delta_1$ RJ on the same circular DNA clone (pCDS67) from fetal thymocytes (data not shown). Although every combination of four distinct gene segments, V, D_1, D_2 and J is possible according to the 12/23 bp spacer rule, a single $D\delta_1$ element has not contributed to V−D or D−J joining and only functioned with $D\delta_2$ as in VDD, DDJ and VDDJ. Together, these data suggest that the D−D joining initiates the adult type of recombination pathway which proceeds as $DD \rightarrow \binom{VDD}{DDJ} \rightarrow VDDJ$. However, in fetal ontogeny a single $D\delta_2$ element can participate in Vδ gene assembly as in $D_2 \rightarrow \binom{VD_2}{D_2J} \rightarrow VD_2J$ (Chien *et al.*, 1987b). The $V \rightarrow VJ$ pathway skipping D segments is observed in common in both fetal and adult patterns of rearrangements. These bifurcated recombination pathways seem to be specific for TCR δ gene rearrangements and are different from the uni-directional sequential model of Ig heavy chain V−D−J joining (DJ → VDJ) (Yancopoulos and Alt, 1985).

Novel $J\delta_2$ usage in thymocytes

Of the two TCR Jδ sequences most of the functional rearrangements detected so far utilize $J\delta_1$, while rearrangements at the $J\delta_2$ locus are relatively infrequent (Chien *et al.*, 1987b). The RJ in clone p016 from circular DNA of adult thymocytes consisted of the 5′ $J\delta_2$ and 3′ V recombination signals (Toda *et al.*, 1988), which provided the first direct evidence for rearrangement involving the $J\delta_2$ locus. In this study, we have shown two more rearrangements at the $J\delta_2$ locus; the reciprocal $V-J\delta_2$ joining in pCDS110 and the $V-D-D-J\delta_2$ CJ in pCDS112. On the other hand, every $J\delta_2$ probe-positive circular DNA clone of adult splenocytes was in germline configuration at the $J\delta_2$ locus. These circular DNAs in mature peripheral T cells may have been derived from α chain rearrangement products. This suggests that some γ/δ T cells utilizing the $J\delta_2$ sequence for gene assembly may emigrate from the thymus with very specific homeing to peripheral lymphoid tissue such as the epidermis (Bonyhadi *et al.*, 1987; Koning *et al.*, 1987) or the intestinal epithelium (Bonneville *et al.*, 1988). Recently, a simplified pattern of $V\delta_1-D\delta_2-J\delta_2$ rearrangement with little or no base insertion has been found in γ/δ-expressing dECs (Asarnow *et al.*, 1988) and γ/δ hybridomas (Korman *et al.*, 1988). Such limited junctional diversity suggests a fetal thymic origin for these cells present in adult mice. Rearrangement at the $J\delta_2$ locus in adult thymocytes is distinct from that in dEC since the former utilized new Vδ subfamilies ($V\delta_7$ and $V\delta_8$) either with the simultaneous participation of two D segments, with the direct V−J joinings skipping the D segments, or with insertion of a long stretch of random sequences at the junction. This rearrangement pattern showing substantial diversity predicts that lymphoid cells utilizing $J\delta_2$, which originated from the adult thymus, may home to peripheral lymphoid organs. Very recently, transcripts of $V\delta_4-D\delta_1-D\delta_2-J\delta_2$ and $V\delta_4-D\delta_2-J\delta_2$ have also been isolated from adult mouse thymocytes but not from splenocytes (Lacy *et al.*, 1989). Transcripts involving the $J\delta_2$ locus have also been found in fetal and adult thymocytes (Ito *et al.*, 1989). However, in α/β-expressing splenocytes excision products contained $V\delta(V\delta_6$ and $V\delta_9)-DD\delta-J\delta_1$ joinings but not $J\delta_2$ rearrangements. This suggests that cells committed to abortive $J\delta_2$ rearrangements cannot switch to TCR α expression. They may die intrathymically or the abortive $J\delta_2$ rearrangements may retard further $V\alpha-J\alpha$ recombination.

Base additions and deletions at junctions

Developing thymocytes showed the adult pattern of δ chain diversity characterized by combinatorial diversity utilizing all Vδ gene segments, by junctional diversity with the simultaneous participation of two D elements, and by random nucleotide insertion (Elliott *et al.*, 1988). Four coding sequences from our characterized circular DNA clones revealed V−D−D−J joints that contained long stretches of inserted N sequences and a signal sequence-derived base at the junctional points. Two other coding sequences, $D_2-J\delta_1$ and $\delta Rec2-D_2-J\delta_1$ also contained a N sequence insertion at the D−J joint. A single D_2 used with N region addition that we considered to be intermediate between the fetal and the adult patterns, suggests that the intermediate event of δRec recombination in the process of differentiation toward the α/β lineage.

Precise heptamer fusion expected for reciprocal recombination products was observed in four joinings of V−Jδ_2 (pCDS110), δRec1−D$_2$ (pCDS73), δRec1−ΨJα (pCDS146) and δRec1−Jα (pCDS144). All RJs of five D−D joinings and one D$_2$−ΨJα joining were imprecisely fused with insertion of germline-encoded or random nucleotides. In the D$_2$−ΨJα RJ (pCDS80), one base was deleted from the 3′ signal heptamer of Dδ_2. A similar deletion has been observed at the RJ of human δRec−ΨJα joining (de Villartay *et al.*, 1988). Although fused heptamers from the residual inversional products of TCR Vβ−Dβ joining (Malissen *et al.*, 1986) and from the excised circular DNAs of TCR Dβ−Jβ joining (Okazaki *et al.*, 1987) showed several residual bases at the joint, random insertional and deletional joining in the fused reciprocal recombination heptamers is without precedent in the endogenous TCR gene rearrangement. Recently, the inclusion of nucleotides derived from signal sequences into Vδ_1−Dδ_2−Jδ_2 rearranged genes has been reported in a dEC (Asarnow *et al.*, 1988) and in a T cell hybridoma (Korman *et al.*, 1988). We reported a single G insertion at the D$_2$−Jδ_2 and δRec2−D$_2$ junctions and a single C insertion at the D$_2$−Jδ_1 junctions that were derived from a signal heptamer (Figures 2b, 3c and 5a). Signal sequence-encoded nucleotides inserted in the CJ may be derived from nucleotides deleted from signal sequences of the RJ. In these cases, cleavage may occur in the signal heptamer. Recently, N region nucleotides have been observed in a RJ generated by inversional rearrangement between Vδ_5 and Dδ_1 (Korman *et al.*, 1989). These imprecise recombination products as found on extra-chromosomal recombination substrates (Lieber *et al.*, 1988) are inconsistent with the general idea that the heptamers are usually fused precisely while the coding partners are not (Alt and Baltimore, 1982). However, precisely fused heptamers were observed in all previously characterized excision products of TCR α gene rearrangements (Fujimoto and Yamagishi, 1987; Okazaki and Sakano, 1988. Toda *et al.*, 1988) and immunoglobulin gene rearrangements (Toda *et al.*, 1989). These data suggest that the RJs of gene rearrangements are precise in TCR α locus but are not always precise in TCR δ locus rearrangements in the same chromosome. This may reflect local differences in chromatin structure or in 'accessibility' of recombinase and terminal deoxynucleotidyl transferase to chromatin of the D region.

Gene rearrangement inactivating TCR δ gene

A new site-specific recombination mediated by δRec and ΨJα has been shown in the human α/δ locus by de Villartay *et al.* (1988). Recombination between δRec and ΨJα could delete the δ locus before productive δ gene rearrangement can occur. These two genetic elements are pseudogenes of V and Jα respectively, but are evolutionarily conserved between human and mouse. Two murine homologs of δRec sequence, δRec1 and δRec2 are identified in the region upstream of Dδ_2. A non-V segment 1.6 kb 3′ of Dδ_1 fused with Dδ_2Jδ_1 (Elliott *et al.*, 1988) was also found to be homologous to these δRecs on the 5′ side of signal heptamer (% homology; 83 to human δRec, 64 to δRec1 and 71 to δRec2) (Figure 6). This may be classified as the third murine homolog of the δRec sequence. The murine homolog of ΨJα is located 3.1 kb upstream of the most upstream Jα (Toda *et al.*, 1988). Although the germline *Eco*RI fragment of δRec1 (8.3 kb) and ΨJα (8.0 kb) were excluded from the circular DNA library due to their size, a significant number of circular DNA clones positive for these probes were scored from 8 week old mouse splenocytes (Table I). This suggests the recombinant structure of each clone. Of 100 circular DNA clones positive for ΨJα−Jα_1 probe, seven clones were positive for Dδ_2−Jδ_1 probe (data not shown), possibly reflecting the presence of Dδ_2−ΨJα joining at significant levels in the circular DNA library. Direct evidence for circular DNA excised by δRec−Dδ_2, Dδ_2−ΨJα and δRec1−ΨJα joining has been obtained by DNA sequencing. ΨJα also recombined with Vα_8 (Toda *et al.*, 1988). When we performed Southern genomic blots of mouse thymus DNA using ΨJα and δRec1 as probes, we found the germline bands became less intense and more complex in day 19 fetal thymocyte DNA and diminished in more mature T cells (data not shown). We have not observed discrete rearranged fragments as shown in human adult thymocytes, possibly due to the diversified pathway of δRec−ΨJα rearrangement. These non-V and non-J signal sequences may render the δ region non-recombinogenic, promoting subsequent rearrangement to the α locus.

Progressive gene rearrangements of the α/δ locus on the same chromosome

Gene rearrangements seem to proceed successively in TCR α/δ locus, as shown by several lines of evidence. First, an average size of the heterogeneous circular DNA of thymocytes measured by electron microscopy is ~17 kb (Yamagishi *et al.*, 1983; Fujimoto and Yamagishi, 1987; Fujimoto *et al.*, 1985), which is far less than the distance spanning Vα and Jα. In splenic T cells, the average size becomes smaller than that of thymocytes (Tsuda *et al.*, 1983). Second, we found that the frequently rearranged Jδ_1 sequence and the infrequently rearranged Jδ_2 sequence were both present at similar frequencies in the circular DNA library made from $\alpha\beta$-bearing T cells (Tables I and IV). Third, the non-functional TCRδ VDDJ CJs found in the circular DNA library from $\alpha\beta$-bearing T cells (Figure 3c) suggest their replacement by succeeding functional TCRα gene rearrangements. Fourth, the frequency of circular DNA clones carrying germline δ sequences in the library is lower than the Jα probe-positive clones, possibly depending on the timing of excisional event and the cycles of DNA replication before the excision. Thus, δ circles excised before α gene rearrangments may have been diluted out during succeeding cell propagation with the occurring further α gene rearrangements. If the majority of circular DNAs came from α chain gene rearrangement products as proposed by Winoto and Baltimore (1989), representation of the δ locus in the library would be higher than that of the α locus. Secondary rearrangements of δ locus on the excised α circles may amplify this tendency. This apparent conflict with our results may be partly due to the nuclear fractionation for preparation of circular DNA they used. Previously, we have found that every circular DNA arises in the nucleus of mammalian cells and the relatively small sized circular DNAs leak to the cytoplasm (Kunisada *et al.*, 1983). Circular excision products of Vα−Jα rearrangements shortened by preceding δ chain gene rearrangements may have been lost from the nuclear fraction. Our results support the progressive gene rearrangement of TCR α/δ locus, modulated by the δ gene inactivating mechanism. Productively rearranged δ locus may be expressed in minor adult thymocyte subpopulations in

association with the functionally rearranged γ chains. All major adult thymocyte subpopulations, which have δ locus non-productively rearranged or deleted by δRec−ΨJα pathway, can undergo further Vα−Jα rearrangements which replace the pre-existing δ locus rearrangements.

Materials and methods

Preparation of circular DNA clone library

Small polydisperse circular DNA molecules were purified from thymocytes and splenocytes of 4 week old and 8 week old C57BL/6 mice, and splenocytes of 8 week old BALB/c-nu/nu mice according to the method described by Fujimoto *et al.* (1985). Since a significant amount of circular DNA is present in cytoplasm (Kunisada *et al.*, 1983), we did not separate the nuclear fraction. They were digested by *Eco*RI and cloned into λgt11 phage vector. The appearance of mitochondrial DNA clones in this library was minimized to ~20% by using a vector with low packaging capacity (up to 7 kb) for mitochondrial *Eco*RI fragments (14.0, 2.1 and 0.2 kb). Recombinant phage titers per μg of *Eco*RI-digested vector DNA were 3.2×10^6 for 4 week old thymocytes, 1.85×10^6 for 8 week old thymocytes, 3.5×10^5 for 4 week old splenocytes, 1.54×10^6 and 5.9×10^5 for 8 week old splenocytes and 1.75×10^6 for 8 week old nu/nu mouse splenocytes. Cloning efficiency into a λZap vector (Stratagene) was decreased to 10^{-2}.

Isolation of germline DNA clones

Genomic DNAs from a C57BL/6 mouse liver were isolated, partially digested with *Eco*RI and cloned into λDASH phage vector. A 1.5 kb *Hin*dIII−*Xba*I fragment of clone p112 (Toda *et al.*, 1988) and a 0.6 kb *Pst*I fragment of pCDS73 (Figure 5) from circular DNA libraries were used as probes to isolate germline clones containing the TCR δ region.

Plaque hybridization and Southern blot hybridization analysis

Plaque hybridizations and Southern blot hybridizations were performed according to the method of Maniatis *et al.* (1982). DNA probes carrying a repetitive sequence were used with driver DNAs. Some of TCR δ probe-positive clones were recloned into pUC19 (Yanisch-Perron *et al.*, 1985) or pHSG399 (Takeshita *et al.*, 1987).

DNA sequence analysis

Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger, 1981) using the universal M13 primer M4, reverse primer RV or appropriate specific primers (see Figure 1, and Table IV footnotes).

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